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Signal Transduction and Actin in the Regulation of G1-Phase Progression

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ABSTRACT: Regulation of cell proliferation is dependent on the integration of signal transduction systems that are activated by external signal molecules, such as growth factors and extracellular matrix components. Dependent on these signal transduction networks, the cells decide in the G1 phase to continue proliferation or, alternatively, to stop cell-cycle progression and undergo apoptosis, differentiation, or quiescence. The MAP kinase and PI-3 kinase pathways have been demonstrated to play an essential role in these G1-phase decisions. Interestingly, actin has been demonstrated to mutually interfere with signal transduction. In addition, it has been indicated that the FOXO transcription factors are involved in these decisions, as well. Actin has been demonstrated to play an important role in the regulation of G1-phase progression. Because of its properties as a structural protein, actin is essential in cytokinesis and in cell spreading and, thus, is involved in G1-phase progression. As an intermediate factor in signal transduction, actin is likely to be involved in cell-cycle regulation induced by external signal molecules. And, finally, actin has been demonstrated to play a direct role in transcription. These observations indicate a prominent role of actin in the regulation of G1-phase progression.

KEY WORDS: ongoing cell cycle, growth factors, cyclins, extracellular matrix, actin-binding proteins, FOXO transcription factors

I. INTRODUCTION

Cell-cycle research has gained enormous attention during the last decades, especially research focusing on the processes underlying the regulation of G1-phase progression. The G1 phase constitutes an important cell-cycle phase, because virtually all nonproliferating cells in an organism contain a G1-phase amount of DNA, indicating that in the G1 phase, decisions are made as to whether the cell continues progression through the cell cycle, or whether this progression is stopped and followed by differentiation programs, induction to apoptosis, or just the establishment of a quiescent status. Thus the G1 phase is characterized by several decision processes. In addition, the G1 phase has been known as a cell-cycle phase in which several checkpoints are active. In these checkpoints, the cells control whether mitosis has been finished properly and whether cells are able to pass S phase properly. Finally, the G1 phase is known for its large morphological changes. During mitosis, the cells are rounded, followed by attachment to the substratum in early G1 phase. After attachment, the cells spread until the flattened well-known morphology has been obtained in mid-G1 phase. These morphological changes are primarily due to actin and the related actin-binding proteins. In this article, we briefly describe the molecular machinery that underlies G1-phase progression, focusing on the interplay between signal transduction complexes activated by soluble signal molecules, such as growth factors, and localized signal molecules, such as attachment factors. Subsequently, we describe the possible role of actin in the processes underlying G1-phase progression, with specific emphasis on actin as a structural protein, as a mediator in cytoplasmic signal transduction, and as a regulator of transcription processes in the nucleus.

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II. REGULATION OF CELL-CYCLE PROGRESSION

Progression through the cell cycle is a well-regulated process that depends upon the interplay between external and internal factors. The external factors, such as growth factors and extracellular matrix components, activate an elaborate intracellular signal transduction network. Subsequently, the signal transduction network ultimately regulates the activities of the cell-cycle engines—the cyclin-CDK complexes (Fig. 1). The precise regulation of the activities of cyclin-CDK results in an orderly sequence of events that constitutes the cell cycle. Because both the intracellular signal transduction networks and the regulation of cyclin-CDK activities have been described in detail in recent reviews,[1–8] we only briefly summarize the main characteristics in this article.

As mentioned above, the engines of the cell cycle are the cyclin-CDK complexes. In these complexes, the cyclins are the activating subunits that interact with specific CDKs to regulate their activity and substrate specificity, whereas the CDKs are serine/threonine kinases that require binding of a specific cyclin in order to be ready to become activated. In addition to binding a cyclin, CDK activity is also dependent on the phosphorylation of threonine and tyrosine residues—some of which are stimulatory, whereas others are inhibitory[9,10]—and on the interaction with specific inhibitory proteins—the CDK inhibitory proteins.[11–13] Two families of CKIs have been identified on the basis of their structures and affinities for cyclin-CDK complexes. The Cip/Kip family, including p21 and p27, influence cyclin-CDK activity by promoting their assembly and/or stabilization. The effect can be either stimulatory or inhibitory.[11] The INK family of inhibitors are specific for the G1-phase CDKs.[11]

The most important mammalian cyclin-CDK complexes known so far are the mitotic cyclins A and B in association with CDC2, and the G1 cyclins D and E in association with CDK4,6 and

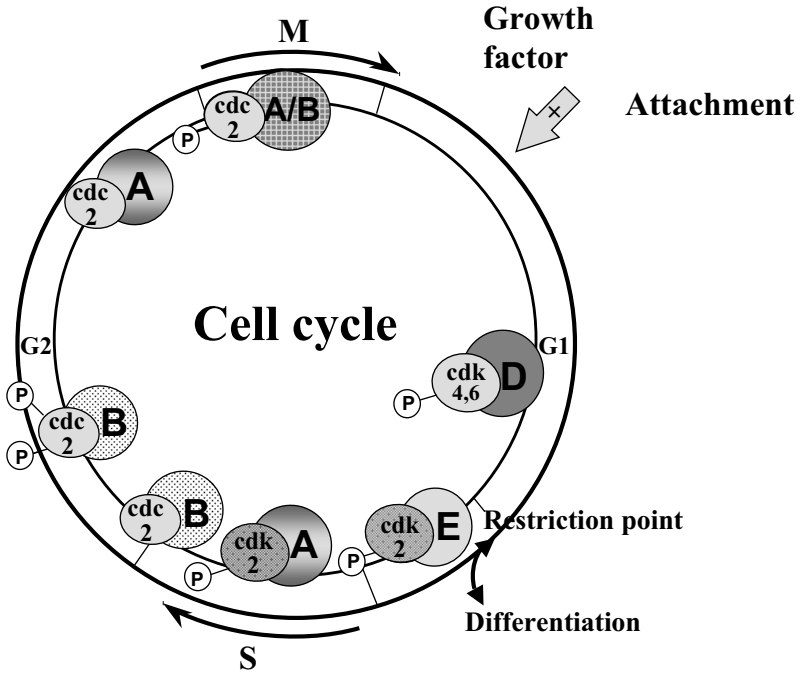

FIGURE 1. Overview of the cell cycle of mammalian cells. The mammalian cell cycle basically consists of four phases: first gap phase (G1), DNA synthesis (S), second gap phase (G2), and mitosis (M). The transition between the different phases is regulated by cyclin/CDK complexes. Different cyclins (A, B, D, E) are present during different cell cycle phases and interact with different CDKs. R is the restriction point defined as the point in the G1 phase after which the cells are independent from external factors for progression of the remainder of the cell cycle.

CDK2, respectively.[10] The first cyclin-CDK complex to be activated during the G1 phase is composed of a D-type cyclin in association with CDK4 or CDK6, depending on the cell type.[14] As cells progress through the G1 phase, cyclin E is synthesized, with a peak late in G1. Cyclin E associates with CDK2 and is important for entry into S phase.[15] Once cells enter S phase, cyclin E is degraded and CDK2 then associates with cyclin A.[16] Finally, cyclin A and the B-type cyclins associate with CDK1 to promote entry into mitosis (Fig. 1). Cyclin A binds to CDK1 with a peak of activity in G2 phase and is then suddenly degraded, whereas entry into mitosis is triggered by cyclin B-CDK1. For exit from mitosis, cyclin B destruction is required.[17]

An important strategy employed in cell-cycle regulation is that one regulatory molecule stimulates one cell-cycle phase and simultaneously inhibits another. Thus, for example, cyclin-CDK activities required for G1/S-phase transitions inhibit the G2/M-phase transition. This strategy ensures that cell-cycle progression is irreversible. This irreversible character of cell-cycle progression is even reinforced by ubiquitin-mediated proteolysis of cyclins once a checkpoint has been passed.[18] Cyclins all encode a PEST sequence, which is recognized by the appropriate F-box protein and targets them for ubiquitination and subsequent proteolytic degradation.[19,20]

One of the most important G1-phase cyclin/CDK substrates in mammalian cells is the product of the retinoblastoma tumor-suppressor gene (pRB).[21] pRB is phosphorylated in a cell-cycle–dependent manner and binds in the hypophosphorylated state to transcription factors, particularly members of the E2F family. E2F consists of at least five different isoforms that form heterodimers with a second group of proteins, known as DP-1.[21] pRB is present in this hypophosphorylated form during early G1 and becomes phosphorylated on several residues during mid- to late G1. This phosphorylation causes the release and activation of the E2F transcription factors, allowing the transcription of genes that mediate progression through S phase.[22] Initial activation of pRB is thought to occur in the G1 phase through phosphorylation by cyclin D/CDK complexes. D-type cyclins can bind directly to pRB in the absence of a kinase and, thus, might target the pRB to CDK4/CDK6 kinases. After the initial phosphorylation by cyclin D/CDK, cyclin E/CDK2 complexes are thought to subsequently phosphorylate pRB late in G1, thereby triggering the onset of S phase.[22]

Another important protein involved in cell-cycle regulation concerns p53. Under normal conditions, the levels of p53 protein are low due to the relatively short half-life of the protein. However, intracellular and extracellular stress signals can induce the stabilization and activation of p53.[23,24] This activation of p53 leads to the transcription of several genes whose products can influence cell-cycle progression, such as the CKI $p21^{Cip1/WAF1}$. Of special interest is the increase in p53 activity upon DNA damage, resulting in cell-cycle arrest and subsequent DNA repair.[24]

III. SIGNAL TRANSDUCTION AND G1-PHASE PROGRESSION

Cell-cycle progression of mammalian cells is largely determined by the action of extracellular signal molecules, such as growth factors and extracellular matrix components. These extracellular signal molecules exert their effects by interaction with specific cell surface located receptors. These receptors have in common that they, upon activation by their ligand, activate an intracellular signal transduction cascade that ultimately results in specific gene expression (Fig. 2). The signal transduction cascades activated by growth factors and extracellular matrix components have been reviewed in detail.[25–28] Therefore, we only briefly summarize these features by describing the most well-known cascades that have been indicated to play an essential role in cell-cycle progression.

A. The MAP Kinase Pathway

One of the most important signaling pathways in the complex network of signal transduction involved in cell-cycle regulation is the mitogen-activated protein kinase (MAPK) pathway (Fig. 2). The MAPK pathway is one of the best-known cascades that is activated by both growth factors and the extracellular matrix. Growth factors bind

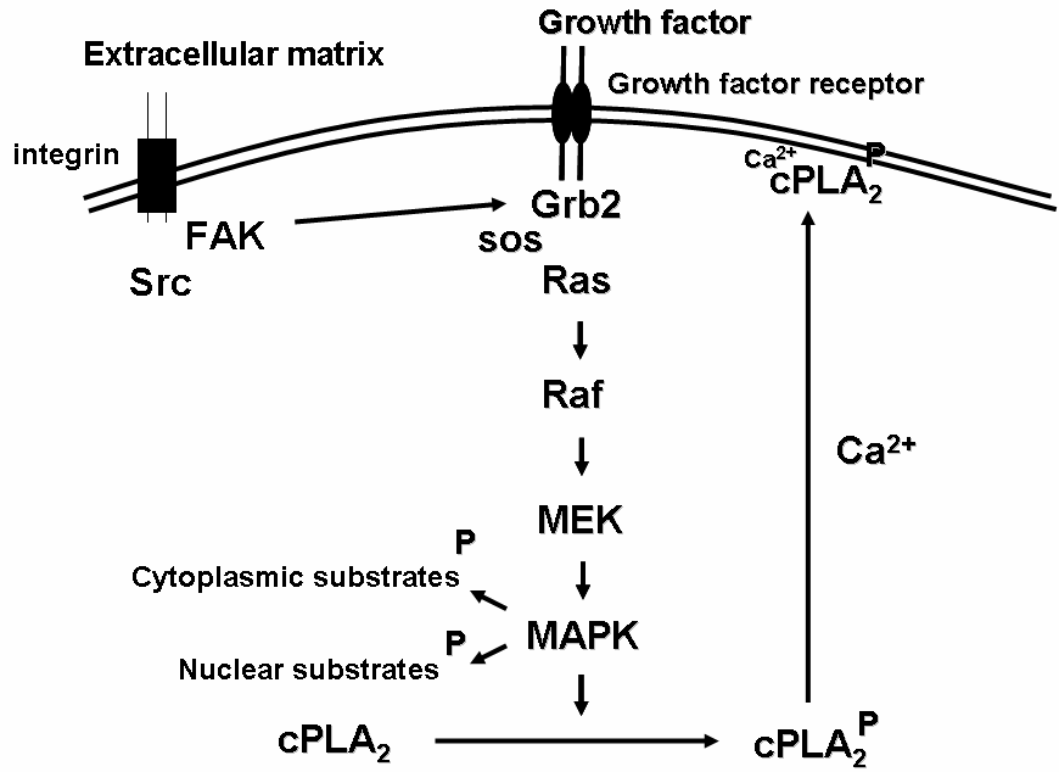

FIGURE 2. Overview of the MAP kinase (ERK1/2) signal transduction pathway. Activation of growth factor receptors or integrins by binding to their respective ligands results in the activation of the small G-protein Ras, which, in its turn, activated the serine/threonine kinase raf. Activated raf phosphorylates and activates the dual-specificity kinase MEK, which, in its turn, phosphorylates MAP kinase on serine and tyrosine, resulting in full activation. MAP kinase phosphorylates several substrates in both cytoplasm (including the cytosolic phospholipase A_2) and nucleus (including several transcription factors).

to their receptors, which are receptor tyrosine kinases. Upon binding, the tyrosine kinase receptors are activated and phosphorylated, thus creating high-affinity binding sites for their substrates. One of the substrates is the adaptor protein Grb2, which, in turn, binds and activates the guanine exchange factor Sos. Sos, in its turn, results in the activation of the G-protein ras. One of the substrates of ras is the serine/threonine kinase raf, which is activated by ras at the plasma membrane. In its turn, raf phosphorylates and activates the dual-specificity kinase MEK, which subsequently phosphorylates and activates the MAP kinase proteins ERK1 and ERK2, as reviewed previously.[29] Similarly, the MAP kinase pathway can be activated by the extracellular matrix (Fig. 2). Upon activation of the extracellular matrix receptors—the integrins, the signal is transmitted to focal adhesion kinase (FAK), also a tyrosine kinase. FAK associates with the cytoplasmic domain of integrins, and upon activation of integrin, the FAK is autophosphorylated. Activated FAK subsequently associates with c-src, a cytoplasmic tyrosine kinase, which further phosphorylates FAK on additional tyrosine residues, leading to full activation of FAK.[30] This phosphorylation results in the binding of the adaptor protein Grb2,[31] which results in the activation of the MAP kinase pathway, as described above for growth factors.[32,33]

The proteins in the MAP kinase cascade that play a central role in cell-cycle regulation are the p42 and p44 MAP kinases ERK2 and ERK1, respectively. ERK1,2 are threonine serine kinases that, upon activation, are able to translocate to the nucleus where specific transcription factors are phosphorylated and activated, such as c-myc,

c-jun, Elk-1, c-Ets-1, and c-Ets-2.[34–37] In addition the activated ERKs can also phosphorylate several cytoplasmic substrates, such as cytoskeletal proteins, cytosolic phospholipase A_2, and others (Fig. 2).[38–41]

Most research done on the role of the MAP kinase pathway in the regulation of cell-cycle progression has been performed by the activation of quiescent cells by mitogens. Upon activation, ERK1/2 translocate to the nucleus where they phosphorylate and activate transcription factors and induce early gene expression.[42] In fibroblasts, activation of the ERK1/2 pathway at the G0/G1 transition has been shown to induce the expression of cyclin D.[43,44] More recently, evidence has been obtained that demonstrates that two waves of growth factor–dependent signaling events are required for progression from the G0 through G1 phase. The first one is an acute burst immediately after growth factor receptor stimulation, and the second one occurs hours after the stimulation.[45] These observations suggest that the first burst of activity is related to the activation of the G0 cells, whereas the second may well be required for G1-phase progression. This latter would be in agreement with our studies on the role of ERK1/2 in the ongoing cell cycle.[46] In these studies, it was demonstrated, using Chinese hamster ovary (CHO) cells synchronized by mitotic cell selection, that p42/44 is phosphorylated immediately after mitosis in early G1 phase. Subsequently, the phosphorylated p42/44 was translocated to the nucleus during the mid-G1 phase, several hours after the initial phosphorylation. Treatment of the cells with an inhibitor of p42/44 phosphorylation in early G1 phase caused a full inhibition of phosphorylation and also inhibition of the nuclear translocation. Furthermore, these cells were unable to progress into S phase, thus demonstrating the necessity of a functional ERK1/2 during progression through G1 phase.[46] Similar conclusions were obtained in fibroblasts in which the ERK1/2 cascade was inhibited by antisense constructs, overexpression of kinase inactive mutants, or inactivation by MAP kinase phosphatase (MKP-1).[47,48]

Although p42/44 appear to play a direct role in progression through the G1 phase of the cell cycle, the downstream pathways also play an essential role. One of the substrates of p42/44 is cytosolic phospholipase A_2 ($cPLA_2$). $cPLA_2$ activity results in the formation of arachidonic acid, and, in turn, arachidonic acid is metabolized into various eicosanoids, including prostaglandins, leukotrienes, thromboxanes, and others. Arachidonic acid and its metabolites have been proposed to play an important role in cell-cycle regulation. Thus, cyclin D1 expression and S-phase entry were induced by prostaglandin F2α in Swiss 3T3 fibroblasts, whereas other prostaglandins were able to arrest cells at the G2/M phase of the cell cycle.[49,50] We have shown that $cPLA_2$ activity increased transiently during mid-G1 phase of the ongoing cell cycle of CHO and neuroblastoma N2A cells, this activity being dependent upon the activity of p42/44.[51,52] By using different inhibitors of $cPLA_2$, it was demonstrated that the activity of $cPLA_2$ in mid-G1 phase was required for entry into S phase. The effects of $cPLA_2$ inhibition on cell-cycle progression were mediated by lipoxygenase rather than cyclooxygenase products, because G1/S transition was only inhibited when lipoxygenase activity was prevented.[52,53]In addition to the ERK1/2 pathways, MAP kinase homologs have been identified in mammalian cells such as the JNK/SAPK and the p38 HOG1 kinase. These MAP kinases respond to distinct extracellular stimuli and have different intracellular substrates. Usually the pathways are involved in specific stress conditions.[54,55]

B. The Phosphatidylinositol 3 Kinase Pathway

Another important signal transduction pathway that plays an essential role in the regulation of cell-cycle progression concerns the phosphatidylinositol 3 kinase (PI 3-kinase) pathway (Fig. 3), because this pathway has been identified as the antiapoptotic pathway. The PI 3-kinase pathway is activated by binding the p85 regulatory subunit of PI 3-kinase to the phosphorylated tyrosine residues of tyrosine kinases, both activated by growth factors and by extracellular matrix components. This recruits the catalytic subunit of PI 3-kinase, p110, to this complex, resulting in the activation of the catalytic domain. Once the p110 subunit of PI 3-kinase is activated, it will catalyze the specific phosphorylation of the inositol ring of phosphoinositides at 3D, generating primarily phosphatidylinositol-3,4,5-triphosphate (PtdIns-

3,4,5-P_3) and phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-P_2). The mechanisms by which PI 3-kinases activate signaling pathways have been recently unraveled. Their lipid products interact with a number of signaling proteins, resulting in membrane targeting and/or modulation of enzyme activity. For example, PtdIns(3,4,5)P_3 binds to a conserved protein motif called the pleckstrin homology (PH) domain, thereby inducing the activation of the serine/threonine kinase Akt/protein kinase B (PKB) and its upstream activators, the phosphoinositide-dependent kinases (PDKs). These kinases phosphorylate a number of substrates, such as $p21^{CIP1}$, Raf, and the forkhead family of transcription factors (FKHR/ AFX/ FOX), which are involved in the control of cell proliferation and survival.[56–58]

Because the PI 3-kinase pathway has been described as the antiapoptotic pathway, the role of PI 3-kinase was determined during the ongoing cell cycle of CHO cells. CHO cells were synchronized by the mitotic shake-off and were subsequently incubated with the specific PI-3 kinase inhibitor wortmannin at several time points after mitosis. The cells were assayed for cell-cycle progression after 24 hours by measuring the [^{3}H]-thymidine incorporation. The addition of wortmannin 4, 6, or 8 hours after mitosis did not cause a significant change in thymidine incorporation as compared to control cells. In contrast, the addition of wortmannin during mitosis, or 2 hours after mitosis, caused a significant decrease of thymidine incorporation. Similar results were obtained with the PI 3-kinase inhibitor LY294002. These observations suggest that PI 3-kinase activity during mitosis and immediately after mitosis is essential for normal cell-cycle progression.[59]

C. Cross Talk Between Growth Factor Receptors and Integrins

Several studies during recent years have indicated cross talk between growth factor receptor tyrosine kinase- and integrin-induced signal transduction cascades.[60–63] Thus, activated growth factor receptors modulate integrin localization and activation, which results in changes in cell adhesion, cell spreading, and cell motility.[64–66] On the other hand, integrin signaling is required for the full activation of growth factor signaling pathways, as

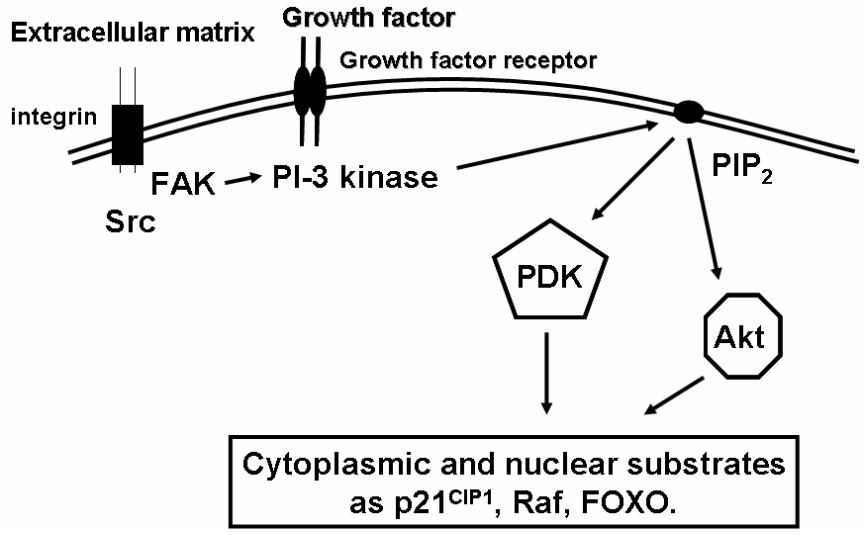

FIGURE 3. Overview of the PI 3-kinase signal transduction pathway. Activation of growth factor receptors or integrins by binding to their respective ligands results in the activation of the PI 3-kinase. This kinase phosphorylates PI on the 3 position in the plasma membrane, resulting in the generation of docking sites for PH-containing proteins, such as Akt. Upon binding the Akt kinase is activated and subsequently phosphorylates cytoplasmic and nuclear substrates. PDK are phosphoinositide-dependent kinases.

deduced from the observations that integrin activation is required for growth factor–induced expression of G1-phase cyclins.[28,67,68] Interestingly, integrins are able to activate receptor tyrosine kinases in the absence of exogenously added receptor ligands, including receptors for EGF, insulin, PDGF, hepatocyte growth factor, vascular endothelial growth factor, and RON.[69–75] This cross talk between integrins and receptor tyrosine kinase receptors is most likely due to the formation of protein complexes between integrins and receptors that allow a direct or indirect interaction.[76–78] In addition to a direct interaction between integrins and receptor tyrosine kinases, downstream interaction between the respective signal transduction cascades has also been reported. For example, in fibroblasts, the loss of integrin-mediated cell anchorage blocks the propagation of the signal from Ras to Raf-1, whereas the activation of ras was not changed.[79] This observation indicates that an anchorage-dependent step exists between Ras and Raf in the signaling cascade triggered by growth factors. A similar anchorage-dependent step has been described to exist between Raf and MEK.[80] These observations indicate the existence of a complex network of signaling proteins that interact on several levels, this interaction being required for optimal signal transmission.

IV. TRANSCRIPTION AND G1-PHASE PROGRESSION

As described briefly above, several signal transduction pathways play an essential role in cell-cycle progression. In addition to the MAP kinase pathway and PI 3-kinase, other essential pathways have also been described, including key proteins such as protein kinase A, protein kinase C, the phospholipases βγ, src kinase, and many small G proteins, such as ras and others. Altogether these observations clearly indicate the existence of an elaborate signaling network in the cell that is highly interconnected, and consequently it is very difficult to pinpoint a specific cascade as being responsible for cell-cycle regulation. However, it is also clear that the ultimate decisions about the fate of the cell are made in the nucleus by specific gene transcription. In this respect the E2F transcription factors have been demonstrated to play an essential role (for review, see Ref. 21). E2F consists of at least five different isoforms that form heterodimers with a second group of proteins known as DP-1.[21] In early G1 phase, E2F is bound to hypophosphorylated pRB. Upon phosphorylation on several residues by cyclin-CDK activity during mid- to late G1, the pRB releases the bound E2F, allowing the transcription of genes that mediate progression through S phase.[22]

However, during the G1 phase, the cells have several decisions to make, ranging from an ongoing proliferation to cell-cycle arrest. In the latter case, the cell-cycle arrest may be followed by differentiation or apoptosis (for review see Refs. 24, 81). Recently, we have demonstrated the presence of two points in the G1 phase of the cell cycle, $G0^-$ and G_R, respectively, in which different decisions are made. $G0^-$ was located very early in G1 phase—immediately after mitosis, and G_R was located at the end of G1 phase. The early restriction point appears to lead to a G0-like state, whereas the second decision point appears to correlate with the restriction point. The entry into the $G0^-$ state is restricted to only a limited period of time after mitosis, whereas entry into G_R phase occurs several hours after mitosis. $G0^-$ was indicated to be related to apoptosis, whereas G_R seems to be related to cell differentiation.[82] Since FOXO transcriptional activity in the nucleus seems to have a crucial impact on the initiation of either quiescence, apoptosis, or differentiation, suggesting that activation of FOXO transcription factors govern different cell fates, depending on whether they occur during $G0^-$ (apoptosis) or G_R phase (differentiation) of the cell cycle.

There are three members of the FOXO subgroup of FOX factors, namely, FOXO1 (FKHR), FOXO3a (FKHR-L1), and FOXO4 (AFX). FOXO regulates the expression of many genes in mammalian cells, whose expression results in markedly different cell fates. For example, activation of FOXO in Chinese Hamster Ovary (CHO) cells can result in cell-cycle arrest and entry into the G0 quiescent stage, whereas T cells and neuronal cells normally respond by the induction of apoptosis.[83] On the one hand, FOXO4 has been shown to inhibit Cyclin D expression and to upregulate p27 expression (CDK4/6 and CDK2

inhibitor), thus resulting in increased protein levels, inhibition of cell growth, and, ultimately, quiescence.[84] On the other hand, FOXO3 has been postulated to trigger apoptosis by inducing the expression of the Fas ligand gene critical for entry into apoptosis.[85] Additionally, FOXO3 has been shown to regulate the expression of proapoptotic Bcl-2 family member Bim in T cells, thus inducing cell death in this cell type.[86] Moreover, FOXO4 is suggested to regulate apoptosis by inducing BCL-6 transcription.[87] This transcriptional repressor, in turn, suppresses the levels of the antiapoptotic BCL-XL protein, thus inducing cell death.[86] Thus, the consequences of FOXO activation are not as clear-cut as suggested by the induction of either cell-cycle arrest (leading to quiescence) or apoptosis. In view of the above-described signal transduction cascades, it is tempting to suggest that the different effects of FOXO activation on cell fate are due to activation of FOXO in different cell-cycle phases by either the PI 3-kinase or the MAP kinase pathway. In agreement with this proposal are the observations that PKB/Akt has been shown to inhibit the transcriptional activity of FOX factors. When located in the nucleus, FOX factors may lead to either apoptosis or exit into G0 (quiescence) phase upon cell-cycle arrest.[83] They are phosphorylated in vivo in the nucleus by PKB/Akt on one threonine and two serine residues.[83] Nuclear phosphorylation of FOXO on Serine 193 in the DNA-binding domain by PKB excludes FOXO from the nucleus and prevents its transcriptional activity.[88] Because FOXO shuttling between the nucleus and the cytoplasm is constitutive and dependent upon RanGTP, Crm1, and importins, phosphorylated FOXO will leave the nucleus, where it will be sequestered by its phosphorylated sites probably by the 14-3-3 protein.[88] As a result, FOXO import into the nucleus is inhibited, its transcriptional activity ceases, and the cells continue proliferating. However, upon serum starvation and in the absence of growth factors, the PI 3-kinase pathway switches off, and, as a result, PKB/Akt can no longer phosphorylate FOXO. This, in turn, enables FOXO to remain in the nucleus and perform its transcriptional activities. Interestingly, preliminary results in our laboratory demonstrate that inhibition of PI 3-kinase during early G1 phase of the ongoing cell cycle results in inhibition of cell-cycle progression, in contrast to inhibition of PI 3-kinase during mid- and late G1 phase.[59]

V. ACTIN AND G1-PHASE PROGRESSION

Actin is an extremely abundant protein in virtually all eukaryotic cells and is involved in many cellular functions, including migration, endocytosis, intracellular transport, docking of proteins and mRNA, attachment, signal transduction, membrane ruffling, neuronal path finding, and cytokinesis. Moreover, it largely determines the cell shape and the position and shape of organelles within the cytoplasm.

The actin family consists of α-, β-, and γ-isoforms. The α-isoform is present in muscle cells, whereas the β- and γ-isoforms are present in all cells. Actin is present in cells in an unassembled, globular form and a polymerized, filamentous form, called G-actin and F-actin, respectively (Fig. 4). The F-actin filaments are composed of two linear strands of polymerized G-actin wound around each other in a helix. Within these filaments, the actin monomers are oriented in the same direction, which causes an inherent polarity of the filaments resulting in the barbed or plus end and the pointed or minus end. The barbed ends are characterized by a rapid polymerization and a slow depolymerization, and the pointed ends exhibit the opposite features. In the cells, actin continuously cycles between the polymer and monomer state, a process called treadmilling.

The actin filaments constitute a highly dynamic network in the cells, the dynamics being regulated by a large number of actin-binding proteins (ABPs).[89,90] The ABPs can be characterized by their function, including cross-linking proteins, actin severing, capping and depolymerizing proteins, monomer-binding proteins, membrane-associated proteins, and actin-regulatory proteins. Several conserved domains of actin have been identified that act as binding domains for the ABPs, including the myosin motor domain,[91] the gelsolin homology domain,[92] the calpain homology (CH) domain,[93] the actin depolymerizing factor/cofilin (ADF/cofilin) domain,[94] and the

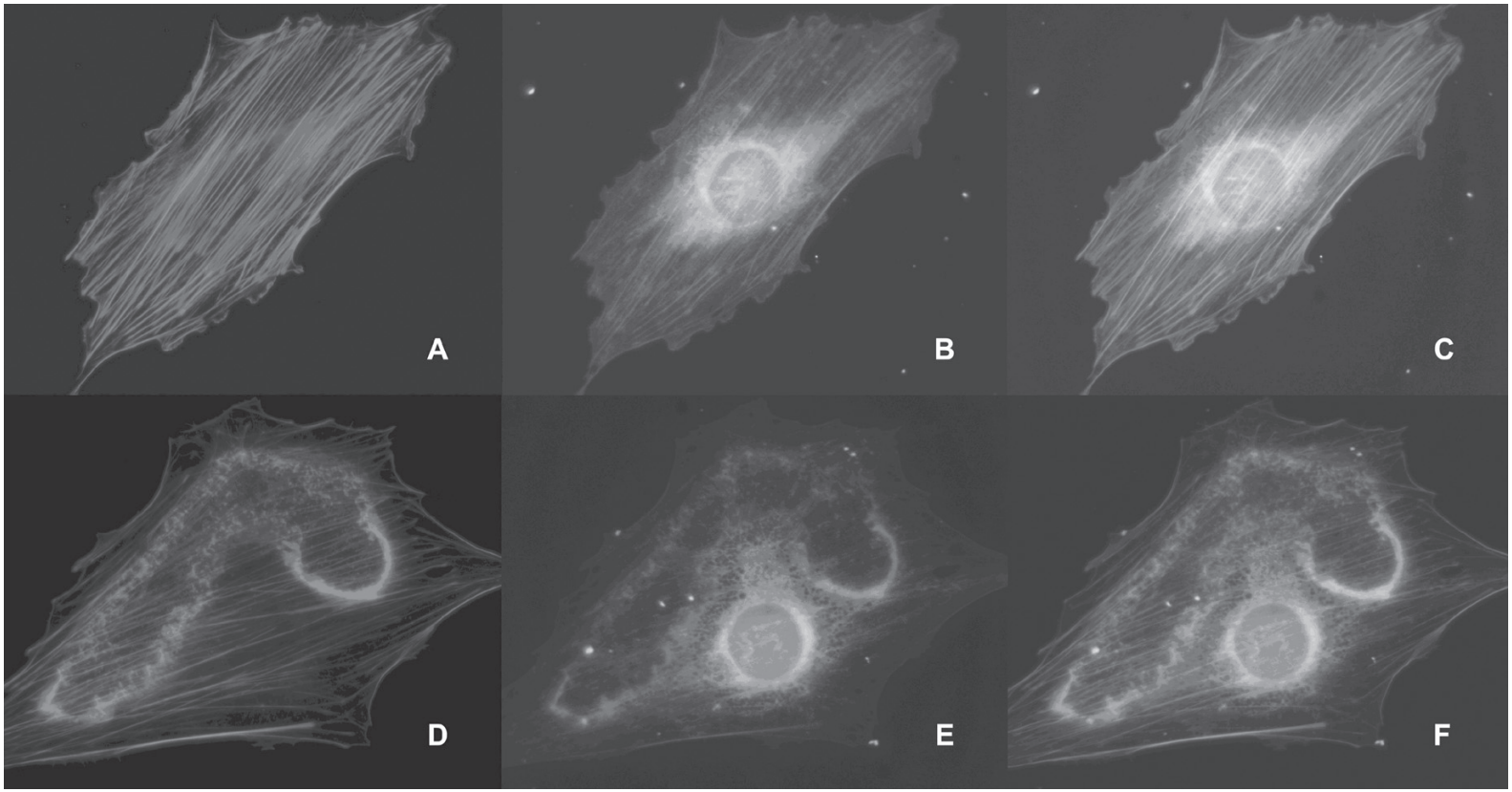

FIGURE 4. Effect of PDGF on F- and G-actin localization in fibroblasts. C3H/10T1/2 fibroblasts were serum deprived for 24 hours and subsequently incubated in the presence or absence of 20 ng/mL PDGF-BB for 10 minutes at 37°. The cells were fixed using formaldehyde and incubated with Phalloidin-Tritc to label F-actin or with DNase I-Alexa488 to label G-actin. F-actin is visible in large stress fibers (**A**), whereas G-actin is localized mainly around and in the nucleus (**B**). Incubation in the presence of PDGF-BB for 10 minutes results in the formation of membrane ruffles and the partial disappearance of stress fibers (**D**). **A** and **B**: control cells labeled for F-actin and G-actin respectively. **D** and **E**: PDGF-treated cells labeled for F-actin and G-actin, respectively. Merged images are presented in (**C**) and (**F**).

Wiskott-Aldrich syndrome protein (WASP)-homology domain-2 (WH2).[95] These observations clearly demonstrate that actin metabolism is regulated by a large number of proteins, which, in their turn, are subject to regulation, as well. This complicated network of actin and the ABPs plays an essential role in cell metabolism and, consequently, also in cell-cycle regulation.

A. Actin as a Structural Protein

Actin is the main constituent of the microfilaments and, as such, plays a dominant role in dynamic cell processes. A direct role of actin in cell-cycle progression concerns its specific activity during cytokinesis (for review see Refs. 96, 97). One of the important processes during cell division is the formation and contraction of the contractile ring. This ring consists of actin-myosin II filaments and a number of ABPs that regulate the actin rearrangements. Among the ABPs are Rho-family small GTPases to regulate the actin polymerization, profilin to regulate actin dynamics, cofilin for actin-filament severing, formin-homology proteins to link Rho signaling to profilin-mediated actin polymerization, caldesmon to regulate myosin II, radixin to cross-link actin to the plasma membrane through CD43, and a number of others.[96] Myosin II motor proteins contribute to the contractility of the cleavage furrow during cell division.[96,97] The actomyosin complex plays an essential role in cell division but is not very important for cell-cycle regulation. Many mutants have been described that lack one or more of the actin-myosin complex, resulting in incomplete or no cell division, but these mutations do not affect the nuclear division. Similarly, inhibition of cell division by actin polymerization–inhibiting compounds, such as cytochalasin, did not influence cell-cycle progression, yielding multinucleated cells.[98,99]

After completion of cytokinesis, cells attach to the substratum, followed by cell spreading in early G1 phase— the latter process again strongly dependent upon actin metabolism (Fig. 5). Cell attachment to ECM components is initiated by the binding of integrins to the ECM proteins, such as fibronectin and laminin. Integrins are heterodimers that are composed of an α and β subunit, each with a large extracellular domain, a single membrane-spanning region, and a short cytoplasmic domain.[29,76] The clustering of integrins is associated with the formation of focal adhesion complexes in cultured cells (Fig 6). These focal adhesions are complex structures containing a variety of structural proteins, such as talin, vinculin, and α-actinin; signaling molecules, such as FAK; and adaptor molecules, such as paxilin, tensin, and $p130^{cas}$.[100,101] Following the activation of focal adhesion proteins by attachment, cell spreading is accomplished with the extension of membrane protrusions, such as lamellipodia and filopodia, and the formation of actin stress fibers (Fig. 6). Activation of the integrins results in the activation of small GTPases of the Rho family of proteins, such as RhoA, Rac1, and Cdc42, the latter two acting as regulators of actin assembly,[102–105] whereas Rho induces focal adhesion and stress fiber formation. Today, on the basis of primary sequence data and known functions, the Rho proteins can be divided into five groups: the Rho-like, the Rac-like, the Cdc42-like, the Rnd, and the RhoBTB subfamilies.[105] The activation of the Rho family proteins by extracellular signal molecules, including growth factors and extracellular matrix components, as well as the downstream effects of the Rho proteins that lead to changes in actin morphology, have been described recently in detail,[105] and the reader is referred for the details and references to this review.

Here, we briefly describe an example in which activation of Rho proteins leads to changes in actin morphology (Fig. 7). In the leading edge, extracellular signal molecules bind to receptors in the plasmamembrane, thereby generating intracellular signaling molecules, such as PIP_2, and activating Rho family GTPases, including Cdc42. Binding of PIP_2 and Cdc42 can subsequently activate WASp/Scar family proteins such as N-WASP. Recently it was described that N-WASP activity is suppressed at PIP_2 levels present in quiescent cells but can be activated by increased PIP_2 levels as obtained in growth factor–stimulated cells.[106] Subsequently, WASP binds to and activates the Arp2/3 complex, which starts to nucleate branched actin filament growth, thus pushing the membrane forward. The elongation of filaments can be terminated by capping proteins such as gelsolin. ADF/Cofilin was shown to

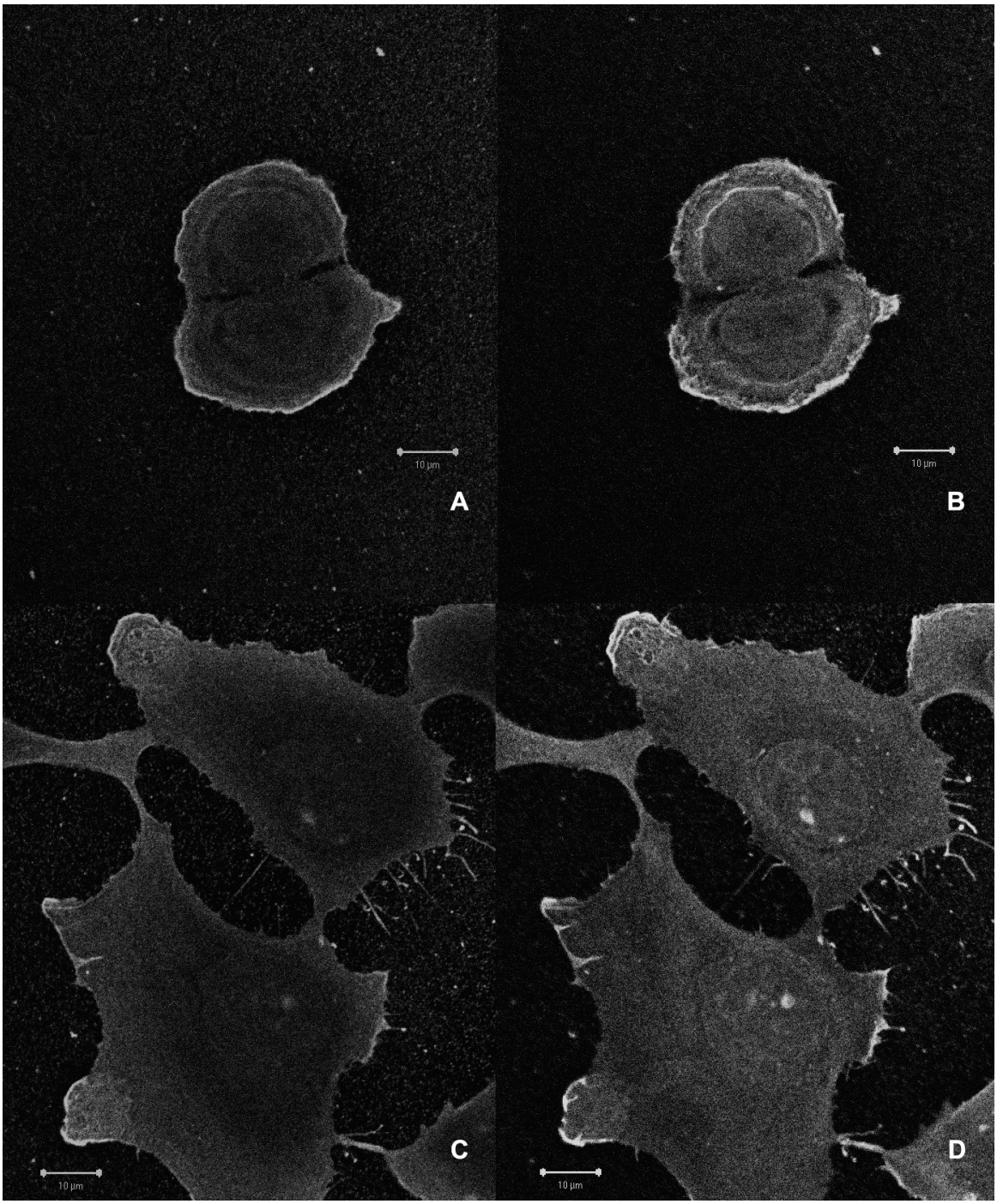

FIGURE 5. β-Actin localization during the G1 phase of HeLa cells. HeLa cells were synchronized by mitotic selection. After synchronization, the cells were plated and cultured for 1 hour (**A** and **B**) or 4 hours (**C** and **D**), fixed with formaldehyde and labeled for β-actin using a monoclonal antibody directed against β-actin (Sigma, A1978, Clone AC-15) and goat-anti-mouse-CY3 secondary antibody. The cells were studied using a confocal scanning light microscopy. Optical sections at 1.48 µm (**A** and **C**) and 2.46 µm (**B** and **D**) from the basal side of the cells, respectively. β-actin is present at the leading edge, in the cytoplasm and in the nucleus of the cells. The scalebar represents 10 µm.

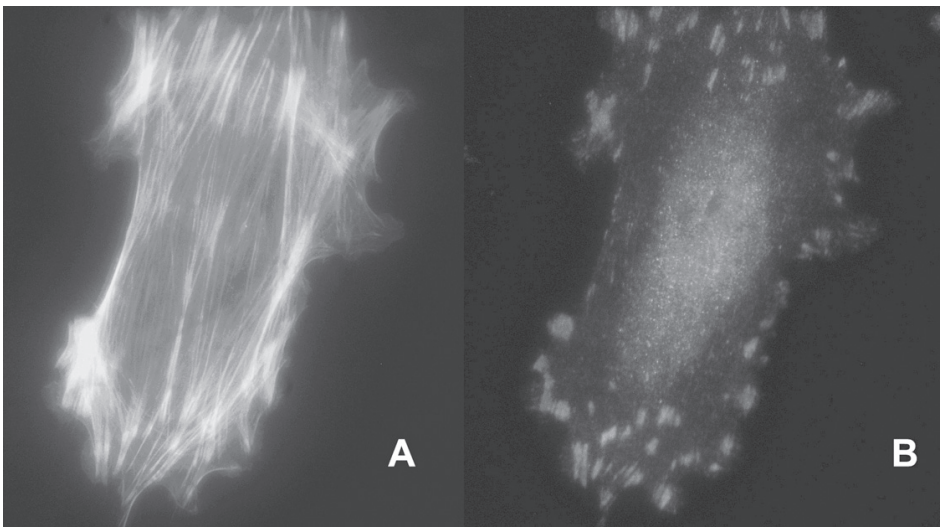

FIGURE 6. Stress fibers and focal contacts in fibroblasts. C3H/10T1/2 fibroblasts were stained for F-actin using phalloidin-Tritc (**A**) and focal adhesion kinase (FAK) phosphorylated on Tyr397 using rabbit antiFAK-pY397 (Biosource) and GARCY3 as a secondary antibody (**B**). The phosphorylated FAK is present in the focal adhesion sites and co-localizes with the F-actin stress fibers.

be involved in creating new free barbed ends and nucleation sites for Arp2/3,[107] resulting in the formation of a branched network of filaments. In the oldest part of a filament, the ATP of each actin subunit is hydrolyzed, and the resulting ADP-actin filaments are severed by ADF/cofilin. The phosphate is released and the resulting ADP-actin dissociates from the filament-pointed ends,

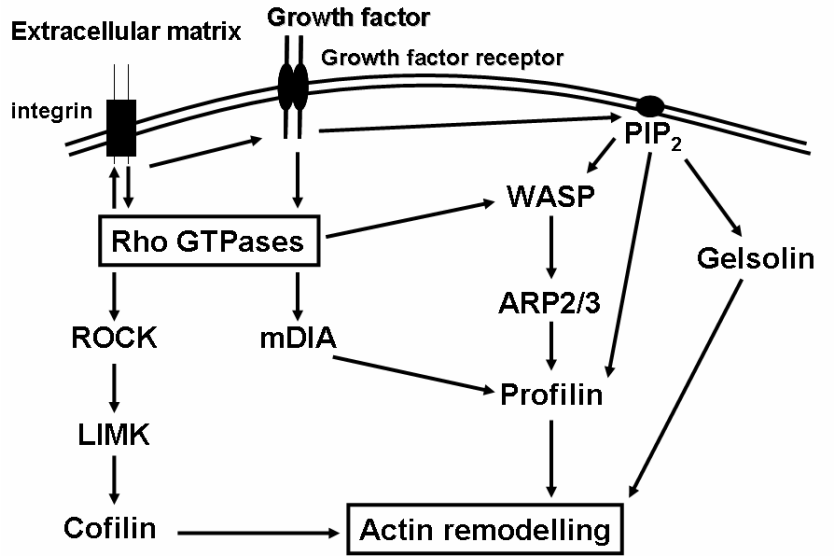

FIGURE 7. Overview of the interaction between signal transduction and actin remodeling. Activation of growth factor receptors or integrins by binding to their respective ligands results in actin remodeling through Rho GTPases. Rho GTPases subsequently activate a kinase cascade including ROCK and LIMK to activate the actin-binding protein cofilin. Alternatively, the profilin is modulated through mDIA or the WASP pathway.

supplying the cell with actin monomers that can now be recycled for new filament formation. Profilin catalyses the exchange of ADP for ATP on the actin monomers, and these can now be used to elongate the last formed filaments at the barbed ends and to form new filaments. Together with thymosin-β4, it maintains a pool of monomeric actin, thereby preventing spontaneous polymerization. Both profilin and cofilin are also under the control of various signaling molecules that result from the same extracellular signaling molecules. So the direction of filament growth is driven by ATP-hydrolysis and can be regulated by extracellular signals.

During the ongoing cell cycle of both CHO and neuroblastoma N2A cells, we have demonstrated that prevention of cell attachment after mitosis caused an arrest of G1-phase progression.[67] Mitotic cells plated on a nonadherent substrate did not attach and no cell spreading was observed. In addition, the cells were not able to progress into the S phase as deduced from thymidine incorporation studies. Interestingly, cyclin D was expressed in these cells, but no cyclin E expression was detected. Plating mitotic cells on a nonadherent substrate coated with poly-L-lysine did result in cell attachment, but no cell spreading was observed. Also, no cyclin E expression was detected in these cells, in contrast to cells plated on the same substratum coated with fibronectin in which a normal G1-phase progression was measured. The results demonstrate that cyclin E expression during the ongoing cell cycle is dependent on cell attachment and subsequent cell spreading induced by integrin activation.[67] These observations suggest that actin polymerization, which is essential for cell spreading, might play an important role in G1-phase progression.

B. Actin as Signal Transduction Mediator

Actin has been demonstrated to be closely related to signal transduction. The first indications for this relationship were obtained by studies on the effect of growth factors on cell morphology. Thus, it was demonstrated that EGF caused the formation of membrane ruffles within minutes after the addition of the growth factor.[108–110] The membrane ruffling was due to actin polymerization. In addition, it was demonstrated that EGF caused actin polymerization in the same time frame as the appearance of the membrane ruffles, whereas both features were completely inhibited by cytochalasin B.[110,111] Similar observations were made on fibroblasts treated with PDGF (Fig. 4). Interestingly, it was demonstrated that abolishment of the actin structure by cytochalasin B caused a super induction of EGF-induced c-fos expression, suggesting that EGF-induced actin polymerization was important for negative feedback regulation of signal transduction by the EGF receptor.[111]

A more close interaction between actin and signal transduction was suggested by the observations that growth factor receptors, among them the EGF receptor, were associated with the cytoskeleton.[112–114] Later, it was demonstrated that the EGF receptor was bound directly to actin.[115] In addition to the receptors, other signal transduction proteins were also found to be associated with the actin microfilaments, including phosphoinositide kinase, diacylglycerol kinase, phospholipase C, Akt/PKB, and others,[116–120] as has been reviewed by Janmey.[121] Altogether, these studies indicated that stimulation of cells with EGF caused a rapid actin polymerization, the formation of membrane ruffles, and the translocation of several of the downstream signaling proteins to these newly formed membrane ruffles, suggesting the formation of signaling complexes at the plasma membrane.[117] The observations summarized above indicate a mutual interaction between signaling cascades and the actin microfilaments, growth factor signaling-induced actin polymerization, and changes in actin morphology, whereas actin, in its turn, regulates signal transduction.

As described above, actin plays an important role in growth factor- and integrin-induced signal transduction. However, both signal transduction pathways are interacting, as well, as exemplified by the ERK pathway. ERK is recruited to focal adhesions in response to several stimuli, such as integrin activation, activation of v-Src, activation of PKCε, and activation of the FGF receptor.[122–124] PDGF and EGF induce cell migration and cause localized cell deadhesion requiring ERK signaling.[125] The effect of growth factors on cell adhesion requires the activation of calpain 2.[126,127] Of particular interest are the observations that calpain activity was decreased in

FAK-deficient cells.[128] In addition, it was demonstrated that FAK induces the formation of a complex constituting calpain 2, FAK, and ERK.[129] These data suggest that FAK is critical to the integration of migratory signals from growth factor receptors and integrins through the ERK pathway to the calpain proteolytic system, resulting in focal adhesion turnover and cell migration.[130]

Actin microfilaments have also been demonstrated to regulate integrins. Treatment of cells with cytochalasin D to cap actin filaments inhibits cell adhesion. In other cells, it was demonstrated that inhibition of actin polymerization resulted in an induction of ligand binding to integrins.[131] Activation of Cdc42 and Rac is associated with the formation of focal complexes in fibroblasts,[132] and inhibition of Rho resulted in a decrease of integrin-mediated aggregation of leukocytes and platelets (reviewed in Ref. 133).

As described above, the signal transduction cascades activated by growth factors and integrins are intimately linked to actin, and, therefore, it seems apparent that actin metabolism itself plays an important role in G1-phase progression, as well. Indeed, disruption of actin architecture with pharmacological agents leads to G1 arrest in a variety of cell types.[134–145] Although cytoskeleton-dependent G1 arrest is related to inhibition of cyclin E expression in Swiss 3T3 cells,[142] most studies report a failure to induce sustained activity of the p42/p44 MAPKs, expression of cyclin D1, and downregulation of the cdk inhibitor $p27^{KIP1}$.[138–141,144] In contrast to the cell-cycle block obtained with pharmacological inhibitors of actin polymerization, inhibition of the Rho-Rho kinase (ROCK) pathway required for stress fiber formation does not prevent the induction of cyclin D1- and G1-phase progression. In fact, inhibition of Rho revealed a cryptic pathway controlled by Rac/Cdc42, resulting in a strikingly early induction of cyclin D1 and accelerated G1-to-S phase transition independent of actin stress fibers and MAPK activation.[146–147] It was proposed that, as long as cyclin D1 is induced, cell-cycle progression is uncoupled from an organized cytoskeleton and the consequent spread cell shape.[146,147] This model is supported by observations that overexpression of cyclin D1 rescues proliferation in nonadherent cells, allowing for anchorage-independent growth as observed in many tumors.[143,148–151] Of particular interest are our observations that disruption of postmitotic actin reorganizations by cytochalasin or latrunculin did prevent cell spreading and the formation of filopodia, lamellipodia, membrane ruffles, and stress fibers but did not influence entry into S phase (unpublished observations). Mitotic cells, as selected by mitotic selection, do express cyclin D, so the results suggest that expression of cyclin D in cells exiting mitosis is sufficient to drive morphology-independent progression through the ongoing cell cycle. In addition, except for endothelial cells and wound fibroblasts, stress fiber formation is not a general feature of cells in living tissue, indicating that proliferation in vivo can and does occur in a stress fiber–independent manner.[152–155]

C. Actin Involved in Transcription

Besides its cytoplasmic localization, actin is also reported to be present in the nucleus (for review see Ref. 156) (Fig. 4). Nuclear actin was implicated to have a role in several processes, including chromatin remodeling, formation of a nucleoskeleton, transport of proteins and mRNA, and transcription. The nuclear localization of actin was demonstrated in various cell types, but often cytoplasmic contamination was seen as the most plausible explanation for the nuclear detection of actin. However, recently, actin was described as a functional component of several nuclear complexes, leaving little doubt about its nuclear presence.[157]

Actin contains two nuclear export sequences (NES) and was shown to be subjected to NES-dependent nuclear export.[158] In addition, a receptor for the export of actin/profilin complexes was identified (exportin 6).[159] Here, profilin was suggested to be a co-factor for nuclear export of actin, whereas nuclear import of actin occurs through binding to cofilin, which contains a NLS. So, it is tempting to suggest that actin is actively kept out of the nucleus to prevent spontaneous polymerization, and cofilin and profilin might play a role in maintaining a balance between the amounts of cytoplasmic and nuclear actin.

In several studies, actin was described to be involved in transcription in direct and indirect ways. Recently, β-actin was shown to have a role in the initiation and continuation of transcription

by RNA polymerase II.[160] Other isoforms of actin were shown to be inactive in transcription. Other studies suggested a functional relationship between nuclear actin and RNA polymerase II.[161,162] An actin-myosin complex associated with RNA polymerase I was described in nucleoli and functionally coupled to elongating transcripts in HeLa cells.[163] Here, an actin-based myosin motor was described to be associated with transcribing ribosomal genes in the nucleus. It was suggested that actin-myosin motors might provide a general mechanism to facilitate elongation of RNA transcripts during transcription of both ribosomal genes and protein-coding genes. Recently, it was indeed demonstrated that both actin and NMI have an essential function in the transcription of ribosomal RNA genes by interaction with the RNA polymerase I machinery.[157] Also a role for β-actin has been described in the transcription by RNA polymerase III.[164] Furthermore, all three RNA polymerase complexes, actin, and profilin were found in Cajal bodies.[165,166] Cajal bodies have been suggested to play a role in the assembly of RNA polymerases[167] and in the maturation of small nuclear ribonucleoproteins.[168]

Actin and profilin were also associated with snRNPs in nuclear speckles.[166,169,170] Interestingly, phosphatidylinositol 4,5-bisphosphate accumulates in these bodies as well as the phosphatidylinositol phosphate kinases (PIPKs).[171] Moreover, the localization of both PIPKs and Ptdlns(4,5)P_2 to speckles was described to reorganize upon inhibition of mRNA transcription, implicating a function for PIP_2 in transcription. In addition, Ptdlns(4,5)P_2 was suggested to be necessary for pre-mRNA splicing and to be present in nuclear particles, whose morphology and distribution was cell-cycle dependent.[172] So there might be a functional relationship in the co-localization of actin, profilin, and PIP_2 in speckles. The question of whether actin, profilin, and PIP_2 play together in a similar way in these bodies, as described in the cytoplasm, is not clear yet.

Besides the presence of profilin in the nucleus, many other ABPs are found in the nucleus—for example, gelsolin, cofilin, and zyxin. Here, they might function in a similar way in the actin metabolism, as occurs in the cytoplasm.

In addition to having a role in transcription, actin and actin-related proteins were implicated to have a role in chromatin remodeling. Actin was shown to be a component of the SWI/SNF-like BAF chromatin-remodeling complex. It might be Ptdlns(4,5)P_2 that couples actin to this complex. In permeabilized nuclei, Ptdlns(4,5)P_2 was able to block the exit of the SWI/SNF-like BAF complex.[173] Moreover, in vitro studies showed PIP_2 binding to the SWI/SNF-like BAF complex, allowing it to associate with actin.[174] So, PIP_2 seems to act as a signaling molecule affecting the function of actin in chromatin remodeling. At present, direct evidence for a role of nuclear actin in regulation of G1-phase progression is lacking, but it seems evident with respect to the possible role of actin in nuclear processes that future experiments will exhibit such a role.

VI. CONCLUSIONS

In this review, we have briefly described the current knowledge on the molecular basis of the regulation of G1-phase progression during the ongoing cell cycle. It is shown that both the MAP kinase pathway and the PI 3-kinase pathway play an essential role in the decisions made in the G1 phase regarding whether the cells continue to proliferate or whether they are programmed for apoptosis or differentiation, respectively. Actin, one of the most abundant proteins in the cells, appears intimately linked to cell-cycle progression, especially during the G1 phase of the cell cycle. This is due to the structural role of actin and, therefore, its role in cytokinesis, cell spreading, and motility. In addition, actin has been demonstrated to be involved in signal transduction from growth factor receptors and from integrins, and the signal transduction cascades and the actin microfilaments have been demonstrated to be mutually linked. Finally, actin is known for its regulatory role in transcription, and on this level, an involvement of actin in regulation of G1-phase progression seems possible. Altogether, these observations indicate that regulation of G1-phase progression is caused by a complex network of signal transduction cascades linked to the complex network comprising actin metabolism. This complicates an analysis of the role of actin in the molecular networks mentioned above concerning the regulation of cell

proliferation. However, a careful analysis with respect to localization and activity during the G1 phase of the ongoing cell cycle will certainly clarify the underlying molecular mechanisms.

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